

## Update on Signaling

# Salicylate, A New Plant Hormone<sup>1</sup>

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Centuries ago, the American Indians and ancient Greeks independently discovered that the leaves and bark of the willow tree cured aches and fevers. It was not until 1828 that Johann Buchner, working in Munich, successfully isolated a tiny amount of salicin, the glucoside of salicyl alcohol, which was the major salicylate in willow bark (for review see ref. 23). The name SA<sup>2</sup>, from the Latin word *Salix* for willow tree, was given to this active ingredient of willow bark by Raffaele Piria in 1838. The first commercial production of synthetic SA began in Germany in 1874. Aspirin, a trade name for acetylsalicylic acid, was introduced by the Bayer Company in 1898 and rapidly became one of world's best-selling drugs. In spite of the fact that the mode of medicinal action of salicylates is a subject of continual debate, they are being used to treat human diseases ranging from the common cold to heart attacks. Because even in aqueous solutions aspirin undergoes spontaneous hydrolysis to SA, the two compounds have similar effects in plants and will be treated together in this review.

Salicylic or *ortho*-hydroxybenzoic acid belongs to a diverse group of plant phenolics. These are compounds with an aromatic ring bearing a hydroxyl group or its functional derivative. The most important mechanism for formation of benzoic acids in plants is the side chain degradation of cinnamic acids, which are important intermediates in the shikimic acid pathway (Fig. 1B). The conversion of cinnamic acid to SA is likely to proceed via benzoic or *ortho*-coumaric acid (1). A recent survey of SA content in the leaves and reproductive structures of 34 plant species confirmed its ubiquitous distribution in plants (14). The highest levels of SA were found in the inflorescences of thermogenic plants and in plants infected with necrotizing pathogens (see below).

### SA AND FLOWERING

The first indication of the flower-inducing effects of SA was obtained in an organogenic tobacco tissue culture supplemented with kinetin and IAA (9), but these observations never attracted much attention because a number of different molecules were found to be active in inducing flower bud

formation in tobacco cell cultures (4). The first suggestion that SA may be involved in the regulation of flowering came from experiments in which aphids were allowed to feed on vegetative and reproductive forms of the short-day plant *Xanthium strumarum*. It was hypothesized that a phloem-transmissible factor responsible for the induction of flowering could be found in the honeydew excreted by aphids. Different fractions of honeydew were tested in a bioassay system using *Lemna gibba* strain G3, a long-day plant, kept in a noninductive light cycle. The flower-inducing substance from *X. strumarum* was identified as SA, which at 5.6  $\mu\text{M}$  caused a maximal induction of *L. gibba* flowering (2). The stimulatory effect of SA on flowering was later demonstrated in other species of Lemnaceae, both short- and long-day, in *Oncidium*, an ornamental orchid species, in *Impatiens balsamina*, a qualitatively short-day plant, in *Arabidopsis thaliana*, and in *Pisita stratiotes* L. (Araceae).

The possibility that SA functions as the endogenous regulator of flowering in *Xanthium*, Lemnaceae, or other plants was diminished by the fact that SA did not induce flowering in *X. strumarum* and that the levels of SA were not different in honeydew collected from vegetative and flowering plants. Also, no changes in the endogenous levels of SA in vegetative or flowering *Lemna* have been reported. In addition, the SA effect was not specific: a large variety of benzoic acids (22), nonphenolic compounds (including chelating agents), ferri-cyanide, nicotinic acid, and cytokinins induced flowering in *Lemna* maintained under a noninductive photoperiod. In a number of species, SA promoted flowering in combination with other regulatory molecules (e.g. gibberellins).

### THERMOGENIC PLANTS AND SEARCH FOR CALORIGEN

Thermogenicity (heat production) in plants, first described by Lamarck in 1778 (8) for the genus *Arum*, is now known to occur in the male reproductive structures of cycads and in the flowers or inflorescences of some angiosperm species belonging to the families Annonaceae, Araceae, Aristolochiaceae, Cyclanthaceae, Nymphaeaceae, and Palmae (11). The heating is believed to be associated with a large increase in the cyanide-insensitive nonphosphorylating electron transport pathway unique to plant mitochondria (7). The increase in the use of this alternative respiratory pathway is so dramatic that oxygen consumption in the inflorescences of *Arum* lilies at the peak of heat production is as high as that of a hummingbird in flight. In addition to activation of the alternative oxidase, thermogenicity involves activation of glyco-

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<sup>2</sup> Abbreviations: SA, salicylic acid; HR, hypersensitive reaction; SAR, systemic acquired resistance; PR, pathogenesis-related; TMV, tobacco mosaic virus.

lytic and Krebs cycle enzymes, which provide substrates for this remarkable metabolic explosion.

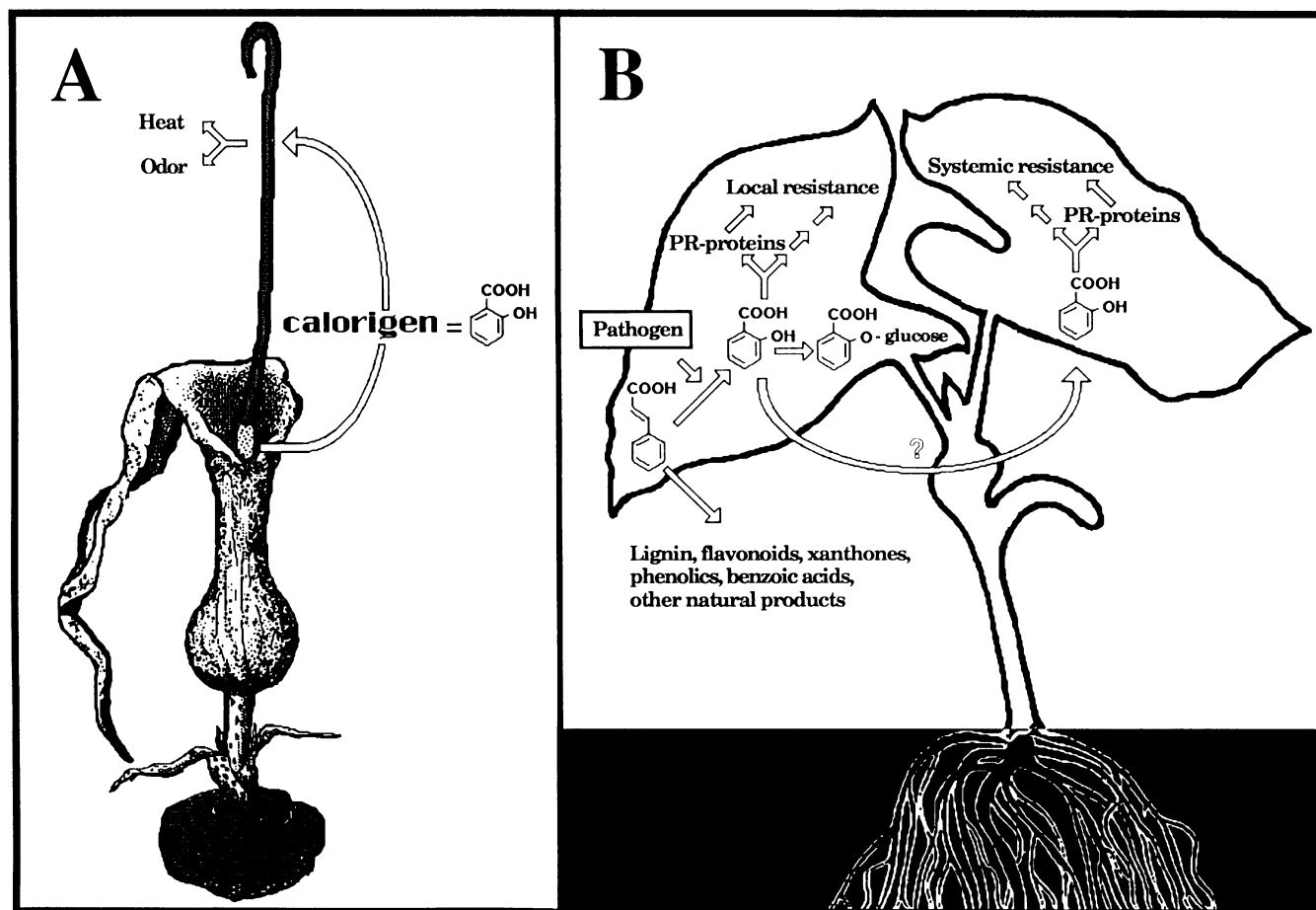
In one of the *Arum* lilies, *Sauromatum guttatum* Schott (voodoo lily), the temperature of the upper part of the spadix known as the appendix increases by 14°C above the ambient temperature on the day of blooming (Fig. 1A). The heat facilitates the volatilization of foul-smelling amines and indoles that are attractive to insect pollinators. In 1937, Van Herk (20) suggested that the burst of metabolic activity in the appendix of the voodoo lily is triggered by "calorigen," a water-soluble substance produced in the male (staminate) flower primordia located just below the appendix.

Van Herk's ideas encountered some skepticism, partially because attempts to isolate and characterize calorigen were not successful until recently. However, in 1987 an attempt to identify the elusive calorigen ended in success. Mass spectroscopic analysis of highly purified calorigen extracted from the male flowers of voodoo lily indicated the presence of SA

(13). Application of SA at 0.13  $\mu\text{g g}^{-1}$  fresh weight to sections of the immature appendix led to temperature increases of as much as 12°C. These increases duplicated the temperature increases produced by the crude calorigen extract both in magnitude and timing, indicating that SA is calorigen. The sensitivity of the appendix tissue to SA increased daily with the approach of anthesis and was controlled by the photoperiod.

On the day preceding the day of blooming, the levels of SA in the appendix of the voodoo lily increased almost 100-fold to 1  $\mu\text{g g}^{-1}$  fresh weight (15). The concentration of SA in the appendix tissue returned to basal, preblooming levels at the end of the thermogenic period. Of 33 analogs of SA tested, only 2,6-dihydroxybenzoic acid and aspirin were thermogenic and induced odor production (15).

The nuclear gene from *S. guttatum* encoding the alternative oxidase protein with an estimated molecular mass of 38.9 kD was recently isolated and characterized (17). Both calorigen



**Figure 1.** Proposed action of salicylic acid in thermogenesis (A) and disease resistance (B). A, On the day before blooming, calorigen, identified as SA, moves from the male flowers of voodoo lily to the appendix. There it induces heat and the production of odor attractive to insect pollinators. The heat is a product of cyanide-insensitive respiration, which, along with the enzymes of the Krebs cycle and glycolysis, is induced by SA. The mechanism by which SA induces thermogenesis still remains a mystery. B, During the development of the hypersensitive response to pathogens, large amounts of SA are produced from cinnamic acid in the vicinity of necrotic lesions. A large portion of the SA is immobilized as  $\beta$ -O-D-glucosylsalicylic acid. Free SA enters the phloem and can be detected in upper leaves. Increases in SA are sufficient for the systemic induction of PR proteins and resistance to subsequent infection. It is still unclear whether or not the export of SA from the infected leaf can account for all of the SA present in the plant during SAR.

extract and SA cause the induction of the alternative oxidase gene, providing additional confirmation of the chemical identity of calorigen. Although the mechanism involved in SA induction of alternative respiration is being unraveled, the mechanism by which SA stimulates glycolysis, the Krebs cycle, and odor production during thermogenesis still remains a mystery.

The discovery of the role of SA in the flowering of thermogenic plants was the first demonstration of an important regulatory role played by endogenous SA. The study ended a 50-year-long search for calorigen and laid the foundation for ongoing investigations of other processes that may be regulated by SA in plants. This discovery also moved SA research from the stage of collecting phenomenological observations to that of making serious attempts to understand the mechanisms of SA action. It is important to remember that both heat and scent production are an integral part of flowering in thermogenic plants. Considering the numerous reports on the induction of flowering by SA (see above), it is tempting to speculate that endogenous SA may play a role in the regulation of certain events in flowering of plants that are not overtly thermogenic.

#### SA AND DISEASE RESISTANCE IN PLANTS

Some disease-resistant plants restrict the spread of fungal, bacterial, or viral pathogens to a small area around the point of initial penetration, where a necrotic lesion appears. This protective cell suicide is referred to as the HR. The HR may lead to SAR, which is defined as a resistance to subsequent pathogen attack that develops in the uninfected, pathogen-free parts of the plant after the initial inoculation (18). SAR develops in a variety of plant interactions with lesion-forming pathogens, is detected several days after the initial infection, can last for several weeks, and is effective against a broad range of pathogens that may be unrelated to the inducing organism.

Commonly associated with HR and SAR is the systemic synthesis of several families of serologically distinct, low mol wt, PR proteins. The localization, timing of appearance, and defense-related functions of at least some PR proteins suggest their possible involvement in SAR. However, definitive proof that the induction of PR proteins causes SAR is still lacking.

It is well established that resistance to pathogens and the production of some PR proteins in plants can be induced by SA or acetylsalicylic acid, even in the absence of pathogenic organisms. The discovery of a protective function of salicylates was made in 1979 in tobacco (*Nicotiana tabacum* cv Xanthi-nc) (24). Xanthi-nc tobacco contains the "N" gene, which originates from *N. glutinosa* and confers HR response to TMV. Salicylate treatments also resulted in the induction of PR-1 proteins in treated leaves. The level of PR protein induction and TMV protection increased with increasing aspirin concentrations. A recent comprehensive study utilizing modern molecular approaches showed that nine classes of PR protein mRNAs that are induced during the development of SAR to TMV in tobacco can be induced by SA to a similar degree (21).

In TMV-susceptible *N. tabacum* containing the recessive "n" allele, TMV does not trigger the induction of PR proteins

and HR. Instead, the virus spreads systemically, causing a characteristic mosaic in younger leaves. However, aspirin induces PR proteins in "n" tobacco and simultaneously reduces the spread and total accumulation of TMV (25). The extent to which SA-induced resistance is based on the induction of PR proteins is still unknown. It is certainly possible that SA activates other resistance mechanisms.

Because SAR can be induced systemically by localized infections, the existence of a systemic signal that activates PR proteins and/or other resistance mechanisms has been hypothesized for at least 25 years (19). Evidence from stem girdling and grafting experiments suggests that the putative signal moves through the phloem tissue of the vascular system of the plant. (6).

The observations that exogenous SA applications induce resistance and PR proteins in plants and that SA is an important endogenous messenger in thermogenic plants, together with the development of analytical methods to quantify its endogenous levels in plant tissues (15), prepared the way to test the possibility that SA is an endogenous messenger that activates important elements of host resistance to pathogens. The single-gene inheritance of TMV resistance in tobacco provided a suitable experimental system in which to investigate this possibility.

A new chapter in SA research started from the observation that SA levels in TMV-resistant (Xanthi-nc), but not susceptible (Xanthi), tobacco increase almost 50-fold, to  $1 \mu\text{g g}^{-1}$  fresh weight, in TMV-inoculated leaves and 10-fold in uninfected leaves of the same plant (10) (Fig. 1B). Induction of PR-1 genes paralleled the rise in SA levels. Although TMV induced PR proteins only in Xanthi-nc tobacco, SA was effective in both Xanthi "n" and Xanthi-nc "N" plants. By feeding SA to excised leaves of Xanthi-nc "NN" tobacco, it was shown that the observed increase in endogenous SA levels is sufficient for the systemic induction of PR-1 proteins (26) and increased resistance to TMV (5). TMV infection becomes systemic and Xanthi-nc plants fail to accumulate PR-1 proteins at 32°C. This loss of HR at high temperature was associated with an inability to accumulate SA. However, spraying leaves with SA induced PR-1 proteins at both 24 and 32°C (26).

SA is also exported from the primary site of infection to the uninfected tissues (26). When leaves of Xanthi-nc tobacco were excised 24 h after TMV inoculation and exudates from the cut petioles were collected, the increase in endogenous SA in TMV-inoculated leaves paralleled SA levels in exudates. Exudation and leaf accumulation of SA were proportional to TMV concentration. Different components of TMV were compared for their ability to induce SA accumulation and exudation: three different aggregate states of coat protein failed to induce SA, but unencapsidated viral RNA elicited SA accumulation in leaves and phloem (26). Mechanical leaf injury did not stimulate SA production and exudation.

The highest concentrations of free SA are observed in and around hypersensitive lesions (5). Chemical and enzymic hydrolysis of extracts from TMV-inoculated leaves demonstrated the presence of an SA conjugate tentatively identified as  $\beta$ -O-D-glucosylsalicylic acid (5) (Fig. 1B). The SA-glucoside was immobile and could be detected only in tissue that contained necrotic lesions. It was not detected in phloem

exudates or virus-free leaves of TMV-inoculated Xanthi-nc tobacco.

Another set of experiments has demonstrated that a fluorescent metabolite identified as SA increased dramatically in the phloem of cucumber plants inoculated with tobacco necrosis virus or the fungal pathogen *Colletotrichum lagenarium* (12). Levels of SA increased transiently after inoculation and reached a peak before SAR was detected. However, analysis of phloem exudate from cucumber leaves demonstrated that the earliest detectable increase in SA occurred 8 h after inoculation with *Pseudomonas syringae* pv *syringae* (16). The systemic accumulation of SA was observed even when the inoculated leaf remained attached to the plant for only 4 h. Although supporting the role of SA as a component of the transduction pathway leading to resistance, these results suggest that another chemical signal may be required for the systemic accumulation of SA in cucumber.

At present, the experimental evidence supports the hypothesis that SA acts as an endogenous signal in induction of PR proteins and at least some components of SAR (Fig. 1B). This conclusion is based on the fact that SA meets the essential criteria of a signal molecule, namely: (a) SA induces resistance to pathogens; (b) SA induces PR proteins; (c) SA levels increase locally and systemically following pathogen attack; and (d) SA moves throughout the plant via phloem.

### CONCLUDING REMARKS

Centuries have passed since the healing substance from willow bark was shown to have value not only for humans but for the plants that synthesize it. Surprisingly, some of the effects of SA in plants are also associated with reduction of disease symptoms. We still do not know if there are any connections between the therapeutic effects of salicylates in plants and animals.

Although all indications are that SA regulates some aspects of disease resistance and thermogenesis, we still do not understand the biochemical link between the action of SA in plant disease resistance and its thermogenic and odor-producing effects in *Arum* lilies. It is also important to elucidate the pathway(s) of SA biosynthesis and metabolism and identify genes involved in these pathways. Furthermore, the molecular components of the SA signal transduction pathway(s) should be elucidated and other possible regulatory functions for SA in plants investigated.

The growing appreciation of the role of SA in plants may bring some practical applications. For example, manipulating the level of SA in plants may be a promising area for the application of biotechnology to crop protection. Increases in endogenous SA may be achieved via enhancing transcription and translation of the genes for SA biosynthesis or by blocking the expression of genes involved in SA metabolism. Engineering transgenic plants with elevated SA levels may be the first step in the creation of crops with increased resistance to agronomically important pathogens.

The classic definition of a plant hormone suggests that it is an organic substance that acts in small quantities at some distance from the site of its synthesis. This definition virtually equates plant and animal hormones. A more recent and probably more universal definition simply states that a plant

hormone is a "natural compound in plants with an ability to affect physiological processes at concentrations far below those where either nutrients or vitamins would affect these processes" (3). All the information on the role of SA in thermogenesis and disease resistance suggests that SA meets these qualifying criteria for a plant hormone.

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